Ammonia and Proton Gated Channel Populations in Trigeminal Ganglion Neurons

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Abstract. The existence of three distinct types of proton-gated sodium currents classified in accordance with their kinetics of desensitization as fast, medium and slow, has been confirmed in the present study on isolated rat trigeminal ganglion neurons. The emphasis was put on the investigation of a peculiar medium type of a chemically gated channel population, using the concentration jump method. The features of the medium-type mechanism allow to distinguish it from the other already known types: it was insensitive to the Ca antagonist verapamil (in contrast to proton-gated sodium current found in dorsal root ganglion neurons), displayed a strong dependence of the kinetics of desensitization on the membrane potential, and (besides the apparent proton-gating) was activated and desensitized by the application of ammonia containing solution at normal pH values. The effect of ammonia itself appeared to be a good tool for the separation of fast and slow proton-gated responses. The results obtained allow to postulate a nonspecific proton-activation of medium-type receptor-channel complexes and their specificity to ammonia ($K_d = 10^{-4}$ mol/l) as an agonist.

Key words: Sensory neurons — Ammonia sensitivity — Proton gating — Sodium current — Olfaction

Introduction

A rapid extracellular increase in proton concentration has been shown to be able to induce an inward sodium current ($I_{Na}(H)$) in isolated sensory neurons (Krishtal and Pidoplichko 1980). Later, $I_{Na}(H)$ observed in chick dorsal root ganglion (DRG) neurons was attributed to the action of proton-modified calcium channels passing sodium current (Konnerth et al. 1987; Davies et al. 1988). At the same time the accumulating evidence for the existence of several different types of proton-gated mechanisms prompted the search for the ways of their separation. The existence of three distinct types of proton-gated sodium currents classified in accordance with

their kinetics of desensitization as fast (F), medium (M), and slow (S) is confirmed in the present study on rat trigeminal ganglion (TG) neurons. The emphasis is put on the investigation of a peculiar medium type of a chemically gated channel population. The characteristics allowing the separation of the medium type channel population from the already known proton-gated types are presented.

Materials and Methods

The experiments were conducted on cells from the TG of 15 to 21 day old rats, dispersed with the help of a simplified "acute isolation procedure" (Ikeda et al. 1986). The cells were kept at 35 °C in the MEM medium (Sigma USA) supplemented with bovine serum (10%), and were transferred if necessary to the intermediate experimental chamber filled with the initial normal solution (NS) with the help of a Pasteur micropipette. The diameter of the neurons taken for experiments varied from 15 to 55 μm. The experiments were carried out at 20–22 °C. The extracellular NS contained (in mmol/l): 150 NaCl; 4 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 HEPES (Serva FRG), pH 7.4; in some experiments a normal basic solution (NBS) with pH 8.4 was used. Normal acidic solution (NAS) with pH 6.2 was employed for the activation of the proton-gated channels. The ionic composition of NS, NBS and NAS was the same. In certain cases 150 mmol/l NH_4Cl were used to replace NaCl. The intracellular perfusing solution contained 130 mmol/l TRIS/HF (Sigma USA), pH 7.2. The extracellular solutions were applied to the cell surface with the help of the "concentration jump" or the "fast hydrodynamical application" technique described elsewhere (Pidoplichko 1986). In all experiments low-pass filtering at 1 kHz was used.

Results and Discussion

The results of the recent investigations of proton-gated sodium currents $I_{Na}(H)$ on isolated rat TG neurons being compared with the findings on other objects (Konnnerth et al. 1987; Davies et al. 1988) suggest the existence of more than one proton-gated mechanism. A helpful auxiliary method of the preliminary estimation of the complexity of $I_{Na}(H)$ is the presentation of $I_{Na}(H)$ trace in semilogarithmic scale. In some experiments on isolated TG neurons fast (F), medium (M), and slow (S) $I_{Na}(H)$ components could clearly be distinguished from each other. (Fig. 1, bottom trace). The semilog scale in this case only emphasizes the separation of the maxima of F, M and S proton-gated sodium currents.

In the majority of cases the integral $I_{Na}(H)$ looked smoothly despite the summing of the three different components with different weights, and at first glance it showed a monoexponential current decay (Fig. 2, a). The most effective way to separate F and S- type proton-gated current types appeared to be the application of the NH_4Cl containing solution which activated and desensitized only the M-type current. It could be effectively visualized as an absent fraction of the integral $I_{Na}(H)$ (Fig. 2,a) using the digital subtraction (DS) procedure (Fig. 2, c). The two-phase (F+S) current seen in Fig. 2 (b) is the result of the application of NAS

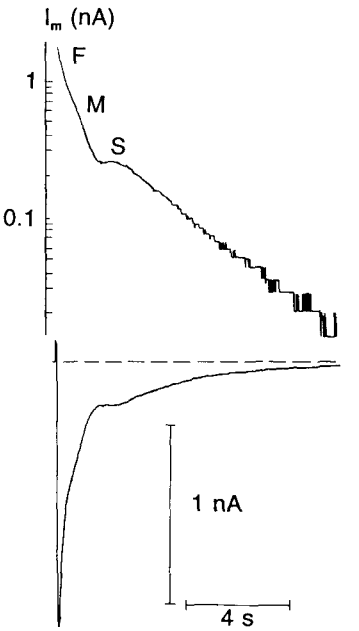

Figure 1. Example of a complex $I_{Na}(H)$. The separation of the tree maxima of the fast, medium and slow currents through the corresponding channel populations is clearly seen. $I_{Na}(H)$ shown at the bottom of the Figure was induced by the transfer of the TG neuron from NS to NAS (see Methods). In the upper part of the Figure the same sum of the F, M and S currents is shown in a semilogarithmic scale. Holding potential (V_h): −60 mV.

after the cell was bathed in 150 mmol/l NH_4Cl solution (pH 7.4).

It has to be stressed that the application of 150 mmol/l NH_4Cl did not activate and/or desensitize F and S responses and vice versa, the proton gating of F and S currents did not depend on the presence of NH_3/NH_4 in the extracellular solution.

The cells with the dominating proton-induced current of a single type were encountered in quite a few cases. In one such cell the demonstration of cross desensitization (by H^+ and NH_3/NH_4 applications) of the M-type currents looked more spectacularly (Fig. 3).

It is clear that the medium-type channels (which apparently were gated by H^+ and displayed a sensitivity to extracellular ammonia) were both selective for Na^+ ions and were passing NH_4^+ ions (Fig. 3, b); thus the corresponding current could be referred to as ammonia-gated ammonium-carried ($I_{NH_4}(NH_3)$). Judging by the amplitudes of the corresponding Na^+ and NH_4^+ currents (Fig. 3) the permeability ratio $P_{NH_4}:P_{Na}$ could be estimated for such M-type channels as being close to 0.5.

Definite evidence for the sensitivity of the M-type currents to extracellular ammonia concentration is illustrated in the Fig. 4*A*. The fact that alkalinization of the external NH_4Cl containing solution increases the extracellular concentration of ammonia was effectively used in electrophysiological experiments (Aickin et al. 1982). The concentration of NH_3 at a given pH value and a known extracellular

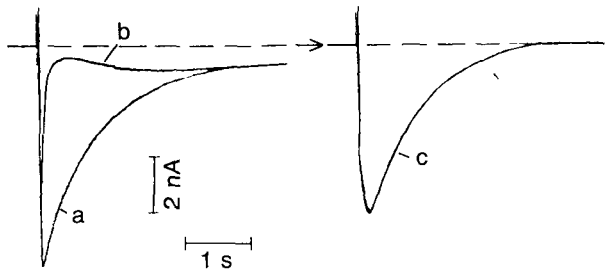

Figure 2. Separation of the medium type inward current. The effect of NH_3/NH_4 application. Left: **a**, the initial integral $I_{Na}(H)$ (which is the sum of the F, M and S responses to the protonation induced by the transfer of the cell from NS to NAS) is compared with **b**, the two-phase (the sum of F and S responses) $I_{Na}(H)$. Prior to the transfer of the cell from NS to NAS the neuron was bathed with 150 mmol/l NH_4Cl containing solution (the response to the NH_3/NH_4 application is not shown). Right: **c**, a full medium-type response activated and desensitized by NH_3/NH_4 application is shown as I_{Na} current (i.e. the absent one due to the completely desensitized fraction of the integral $I_{Na}(H)$ visualized as the difference between traces "**a**" and "**b**") as evaluated by the digital subtraction (DS) procedure (a). $V_h = -60$ mV. Desensitization time constant (τ_d) is 780 ms.

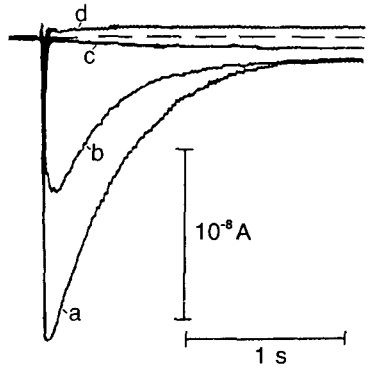

Figure 3. Cross-desensitization of $I_{Na}(H)$ and the ammonia-gated $I_{NH_4}(NH_3)$ M-type currents. **a.** The initial $I_{Na}(H)$ current (M-type predomination) due to the transfer of the cell from NS to NAS. **b.** $I_{NH_4}(NH_3)$ current evoked by the application of the 150 mmol/l NH_4Cl containing solution. **c.** The absence of the M current due to the transfer of the TG neuron from 150 mmol/l NH_4Cl to NAS. **d.** The absence of the M current due to the transfer of the cell from NAS to the acidic 150 mmol/l NH_4Cl containing solution (pH 6.2). $V_h = -70$ mV. The M-type τ_d is the same for the M-type $I_{Na}(H)$ and M-type $I_{NH_4}(NH_3)$, being about 560 ms.

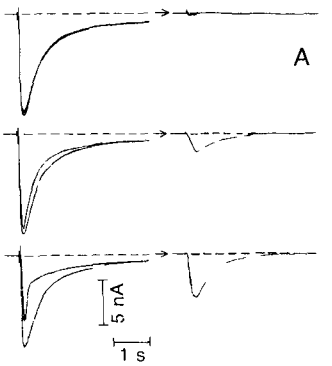

Figure 4 *A*. The ammonia activation and desensitization of the M-type channel population. The dependence of the M-type current on the extracellular ammonia concentration. Upper traces, left: $I_{Na}(H)$ (which is a sum of F, M and S responses and is induced by the transfer of the cell from NS to NAS) is compared with a similar current elicited by the transfer of the same cell from NBS to NAS. The trace at right is a result of digital subtraction of one response from another, illustrating the absence of any significant changes in $I_{Na}(H)$ upon the transfer of the cell from the more alkaline solution to the NAS. Middle traces, left: the initial $I_{Na}(H)$ is compared with $I_{Na}(H)$ of a smaller amplitude induced by the transfer of the neuron from 5 mmol/l NH_4Cl containing NS (i.e. 3.4×10^{-5} mol/l $[NH_3]_{ext}$ calculated for 25 °C at pH 7.4) to ammonia/ammonium-free NAS. Right: the fraction of the M-type current (activated and desensitized during the application and washing of the neuron in 3.4×10^{-5} mol/l NH_3-containing solution) is evaluated using the DS procedure (a). The response to the application of the NH_3-containing solution is not shown. Lower traces, left: the initial $I_{Na}(H)$ evoked by the transfer of the neuron from NBS to NAS is compared with the diminished $I_{Na}(H)$ of a complex shape (a sum of F, diminished M and S currents) induced by the transfer of the cell from the 5 mmol/l NH_4Cl-containing NBS (i.e. 3.2×10^{-4} mol/l $[NH_3]_{ext}$ calculated for 25 °C at pH 8.4). Right: the increased fraction of the M-type response which was activated and desensitized in the solution with the higher (due to alkalinization) extracellular NH_3 concentration is evaluated with the help of the DS procedure (a). The holding potential was the same for all parts of the Figure (−80 mV). The process of desensitization of the M-type current (obtained in the net form due to the DS procedure) is monoexponential with a time constant of 520 ms.

concentration of NH_4Cl is given by the formula:

$$[NH_3]_{ext} = \frac{[NH_4Cl]_{ext}}{1 + 10^{pK_a - pH}} \quad (1)$$

where pK_a is calculated for 25 °C (Bates and Pinching 1950) and is equal to 9.24. Thus, at 5 mmol/l NH_4Cl in the NS at pH 7.4 (i. e. 3.3×10^{-5} mol/l $[NH_3]_{ext}$)., the fraction of the M-type current activated and desensitized by the application of this solution was comparatively small (as evaluated by a DS procedure after the transfer of the TG neuron form ammonia containing NS to NAS, Fig. 4*A*, middle traces, a), and was much more pronounced upon increasing the extracellular concentration of NH_3 by about an order of magnitude (3.2×10^{-4} mol/l $[NH_3]_{ext}$), due to the alkalinization (pH 8.4) of the extracellular solution (Fig. 4*A*, bottom traces, a). The dose-response curve for such an effect (Fig. 4*B*) of extracellular

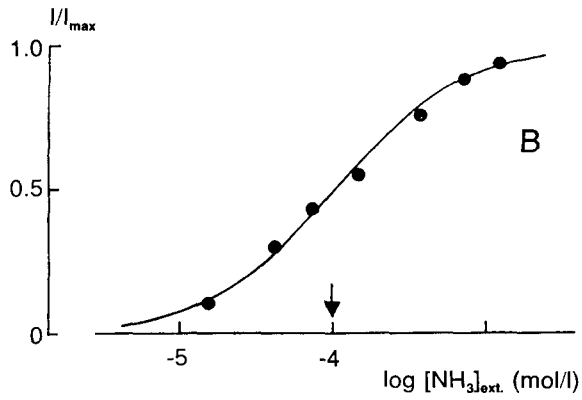

Figure 4 *B.* A dose-response curve for the inward sodium currents through ammonia-sensitive channel population with the medium kinetics of desensitization. The currents were obtained by the digital subtraction procedure. The free ammonia concentration in the extracellular NH_4Cl-containing solution was calculated for $pK_a = 9.24$ at pH 7.4 and 25 °C. The solid line is marking the Langmuir isotherm and has been drawn through the experimental points with $K_d = 10^{-4}$ mol/l $[NH_3]_{ext}$ (marked by the arrow). Ordinate: the normalized value of the M-type current. Abscissa: the common logarithm of the ammonia concentration in the extracellular solution $V_h = -80$ mV.

ammonia concentration was obtained for another cell at pH 7.4 by a DS procedure after the M-type current fraction was activated and desensitized by the application of extracellular solutions with different NH_4Cl concentrations (NaCl was replaced by TRIS (HCl)); the absent fraction of the M-type current was visualized by the transfer of the cell from the NH_4Cl-TRIS containing solution to NAS, and the subsequent DS procedure. The $[NH_3]_{ext}$ was calculated by formula (1). The curve fitted a Langmuir isotherm with a K_d of about 10^{-4} mol/l.

The NH_4^+:Na^+ permeability ratio for M-type channels was also estimated in a more precise way from the changes in the reversal potential (E_{rev}) of the corresponding currents, according to the equation (Dwyer et al. 1980):

$$\Delta E_{rev} = E_{rev\ NH_4,Na} - E_{rev.Na} = 25 \ln \left(1 + \frac{P_{NH_4}[NH_4]_{ext}}{P_{Na}[Na]_{ext}} \right) \qquad (2)$$

In these experiments (Fig. 5) the TG neuron was perfused intracellularly with the solution containing 65 mmol/l NaF and 65 mmol/l TRIS (HF). Extracellular solution contained (in mmol/l: 75 NH_4Cl; 75 NaCl; 75 TRIS (HCl) and 75 NaCl; or 50 NH_4Cl and 100 NaCl; 50 TRIS (HCl) and 100 NaCl.

At 75 mmol/l Na in the extracellular solution, $E_{rev\ Na}$ of the M-type current was 4 mV ($E_{rev\ Na\ theor} = 3.6$ mV), and at 100 mmol/l$[Na^+]_{ext}$ it was equal to

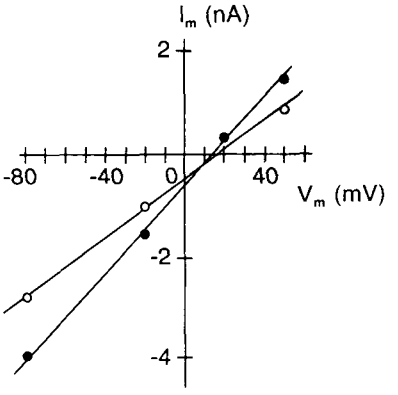

Figure 5. Estimation of the reversal potential of the medium current in Na^+ and NH_4^+ containing solution. Filled circles: experimental points marking the magnitude of the M-type current at different holding potentials. The reversal potential was determined for the extracellular solution containing 75 mmol/l NH_4Cl and 75 mmol/l NaCl as being 14 mV. Open circles: experimental points for the determination of the reversal potential of the M-type current in 50 mmol/l NH_4Cl and 100 mmol/l NaCl containing extracellular solution. E_{rev} = 17 mV. In both cases the intracellular solution contained 65 mmol/l Na^+. The experiment was conducted on the same cell showing the predominating M-type current.

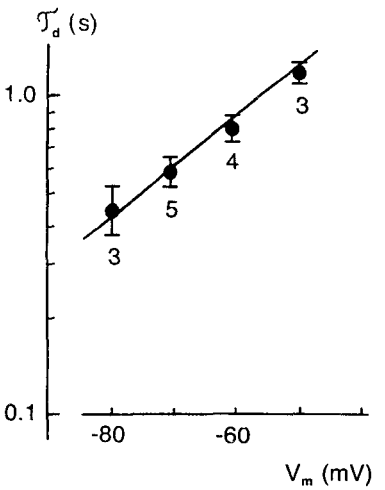

Figure 6. The dependence of τ_d of medium-type currents on the membrane potential. The figures at the experimental points indicate the number of M-type τ_d values averaged for a given V_h. M-type currents were recorded from different cells. Ordinate: τ_d values in milliseconds, log scale. Abscissa: membrane potential in millivolts.

11 mV ($E_{rev\ Na\ theor}$ = 10.8 mV). In extracellular solution containing 75 mmol/l NH_4 and 75 mmol/l Na, the M-type current reversed at about 13 or 14 mV, and in 50 mmol/l NH_4 and 100 mmol/l Na solution the E_{rev} was about 17 mV. Thus, the P_{NH_4}: P_{Na} ratio calculated in accordance with equation (2) was close to 0.5 and agreed with the result of the preliminary estimation (Fig. 3).

The process of desensitization of the M-type current (evaluated by a DS procedure) was always monoexponential and displayed a pronounced dependence on the potential difference across the membrane. The dependence of the time constant

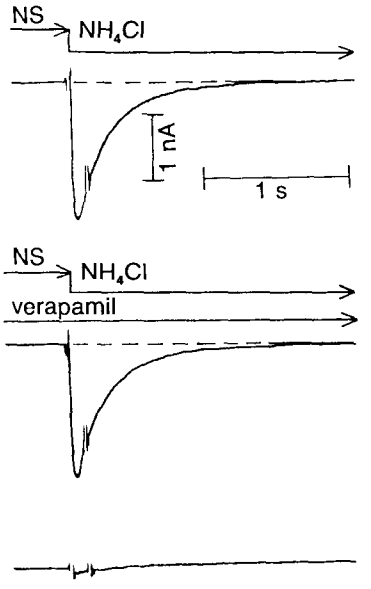

Figure 7A. The insensitivity of the M-type currents to verapamil. The absence of verapamil action on $I_{NH_4}(NH_3)$. The upper trace is the inward current carried by NH_4 ions and elicited by the transfer of the TG neuron from NS to 150 mmol/l NH_4Cl solution (pH 7.4). The horizontal lines above the trace indicate the moment of the application of a new solution and the duration of the application. Middle trace: the same inward current in the presence of 100 μmol/l verapamil added into both solutions (indicated by a second horizontal line). The lower trace is the result of the digital subtraction of one trace from another, demonstrating the absence of verapamil action. The artifacts of electromagnetic valve switching are clearly seen. $V_h = -100$mV, $\tau_d =$ 245 ms.

of the M-type desensitization (τ_d) on the membrane potential is shown in Fig. 6. Upon a 25 mV shift in the hyperpolarizing direction τ_d was speeding up e-times.

Recently Davies et al. (1988) investigating $I_{Na}(H)$ in DRG neurons postulated the possibility of the modification of electrically gated Ca^{2+} channels by protonation (thus transforming them into Na ligand-gated). The evidence presented in favor of this suggestion included the blocking action of Ca^{2+} channel antagonists on $I_{Na}(H)$ in DRG neurons.

In our case, the possibility of Ca^{2+} channel modification can be ruled out. First; the TG neurons being perfused with fluorine anions never displayed electrically gated Ca^{2+} currents. Second; in the present experiments the action of 100 μmol/l verapamil was checked on the peculiar M-type current gated by an increased extracellular proton concentration or by NH_3/NH_4 containing extracellular solutions. For such experiments the neuron was chosen which responded to protonation with the predominating M-type current only. The absence of verapamil action on the M-type current activated by the transfer of the neuron from the NS containing 150 mmol/l NaCl to the solution containing 150 mmol/l NH_4Cl is shown

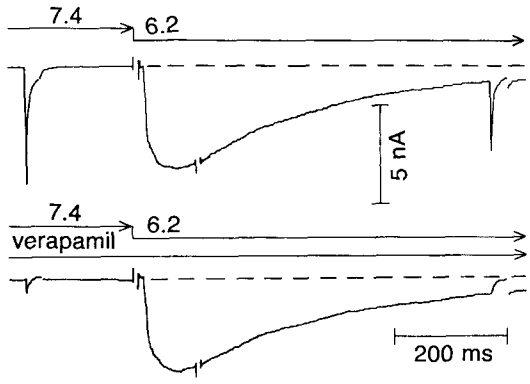

Figure 7*B*. The insensitivity of M-type $I_{Na}(H)$ to verapamil. The same cell as in Fig. 7*A*. Upper trace: sodium TTX-sensitive currents flowing through the electrically gated channels (the capacitive transients have been compensated for with the help of the analog circuit), and the predominating M-type $I_{Na}(H)$ are displayed on the same time scale. The horizontal lines above the traces are indicating the moment of the exchange of NAS for NS. Lower trace: the same procedure except that 100 μmol/l verapamil were added in both solutions. The durations of the verapamil action is indicated by the second horizontal line. $V_h = -100$ mV, testing potential (V_t) $= -30$ mV.

in Fig. 7*A*. (In this case, the current was carried by NH_4^+ ions.) On the same cell the action of verapamil was investigated upon NAS application (Fig. 7*B*, note the different time scale). The predominating M-type $I_{Na}(H)$ was practically unaffected by 100 μmol/l verapamil, in contrast to the blocking action of verapamil on electrically gated TTX-sensitive sodium current. (The nonspecificity of verapamil as calcium antagonist for mammalian tissues has already been demonstrated in rat cardiomyocytes (Pidoplichko and Verkhratsky 1989)).

Judging by the data presented above we may conclude that the population of M-type channels observed differs from the already known mechanisms. We should like to point to a paradox: as a matter of fact, the apparent proton-gated channel population with the medium-type kinetics of desensitization is the ammonia-sensing mechanism which can respond to the elevation of the ammonia concentration at normal and alkaline pH values by the inward sodium current. One tentative suggestion which may be helpful in solving the paradox may be that the peculiar receptor-M-type channel complex is somehow structurally associated with that of the actual proton-gated channels: the fast one, the slow one or both. It may then be that the proton gating of the fast-type channels or the slow-type channels or both at one time forces the gates of the medium-type (in fact the ammonia-sensing) channels to respond and to undergo conformational changes required to open the ion-passing pore selective for sodium ions.

Another problem is the physiological significance of the observed ammonia-sensing mechanism. The concentration of ammonia in the blood in normal conditions can be of the same order of magnitude (Fig. 4*B*) sufficient for complete steady state desensitization of the medium type channels. Since such a mechanism was found in the membrane of trigeminal ganglion neurons it may be assumed (if it is actually the ammonia sensor and no better agonist is found) that such receptor-channel complexes can be incorporated in the membrane of free nerve endings participating in the primary sensory mechanisms of olfaction.

Apart from the more or less probable suggestions concerning the M-type ligand-gated mechanism the evidence reported can inspire new lines of investigations and also provide an effective instrument for the separation of proton-gated channel populations allowing their detailed characterization.

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